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Immunohistochemical characterization of transgenic mice highly expressing human lysosomal α-galactosidase

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Abstract

Human lysosomal α-galactosidase predominantly hydrolyzes ceramide trihexoside. A transgenic mouse line, C57BL/6CrSIc–TgN(GLA) 1951 Rin, highly expressing human α-galactosidase, has been established and investigated biochemically and immunohistochemically in order to clarify the distribution of the expressed enzyme proteins and to evaluate it as a donor model of organ transplantation therapy for Fabry disease caused by a genetic defect of α-galactosidase. In these transgenic mice, about five copies of the transgene were integrated, and α-galactosidase activity was expressed in liver, kidney, heart, spleen, small intestine, submaxillary gland, skeletal muscle, cerebrum, cerebellum, bone marrow cells and serum. The enzyme activity was about 22 to 11,080-fold higher than that in non-transgenic mice. In liver, heart and kidney tissues, which are important organs for transplantation studies, sufficient amounts of α-galactosidase mRNAs were transcribed, and the expressed enzymes, with molecular weights of 54–60 kDa, are abundant in the liver (enzyme activity: 53,965 nmol h^{-1} mg^{-1} protein) and heart (39,906 nmol h^{-1} mg^{-1} protein), followed by in the kidney tissue (9177 nmol h^{-1} mg^{-1} protein), respectively. An immunohistochemical microscopic study clearly demonstrated the distribution of the expressed enzyme proteins in kidney and liver tissues. Highly expressed α-galactosidase was detected in glomerular cells, tubular cells and hepatocytes. These transgenic mice will be useful as a donor model for experimental organ transplantation, and also it will enable recurrent biopsies and long-term observation. The organ transplantation data on mice will provide us with important information.

Keywords: α-Galactosidase; Transgenic mouse; Fabry disease; Tissue transplantation

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1. Introduction

Lysosomal α-galactosidase (EC 3.2.1.22) catalyzes the degradation of ceramide trihexoside. The gene encoding α-galactosidase has the structural organization and regulatory elements of a housekeeping gene, and the enzyme protein is expected to be expressed in many tissues and organs. But the details of its distribution have not been clarified yet. A genetic defect of the enzyme causes Fabry disease, and results in systemic accumulation of the substrate, especially in the kidneys, heart and vascular endothelium [1]. In patients with Fabry disease, a renal disorder develops in adolescence and determines the prognoses of patients.

So far, infusions of plasma, leukocytes and purified α-galactosidase [2,3] and plasma exchange [4] have been tried as therapies for this disease. But, unfortunately, stable clinical effects have not been obtained. Other attempts were made, including transplantation of kidneys [5] and fetal livers [6]. In the case of renal transplantation, the early experience was not favorable [7,8]. However, successful transplantation has been achieved recently [9–11], and long term observation is necessary. As to fetal liver transplantation, only two cases have been reported, but the long term prognosis is not clear.

We have established transgenic mice carrying human α-galactosidase in many tissues, bone marrow cells and serum. In this study, we performed biochemical and immunohistochemical analyses of the kidney and liver tissues, which are useful organs for transplantation studies, and succeeded in detecting the expressed human α-galactosidase immunohistochemically and in clarifying its distribution in these tissues. These results and further experimental transplantation studies involving the transgenic mice as a donor will provide us with useful information for the improvement of organ transplantation in humans.

2. Materials and methods

2.1. Transgenic mice

Transgenic mice carrying human α-galactosidase cDNA were established in our laboratory, according to the methods described previously [12]. Briefly, α-galactosidase cDNA was prepared from cultured lymphoblast poly(A)$^+$RNA by means of the reverse transcription-polymerase chain reaction (PCR) [13]. The cDNA encoding human α-galactosidase was inserted in the vector, pCXN2 (supplied by Dr. J. Miyazaki, Tohoku University, Japan) [14], and then a 3-kb DNA fragment including the chicken β-actin promotor and human α-galactosidase cDNA, produced by *Nde*I digestion of the construct (produced by Dr. S. Ishii, Usuki Bioresearch Center, Japan), was microinjected into pronuclei of fertilized eggs from superovulated C57BL/6CrSIc female mice according to the standard method [15]. Then, the zygotes were implanted into pseudopregnant female SIc:ICR mice. The transgenic mice were designated as C57BL/6CrSIc–TgN(GLA)1951Rin, abbreviated as TgN(GLA)1951. Animal experiments were performed according to local institutional guidelines.

2.2. Southern blot analysis

Genomic DNA was extracted from the livers of transgenic and non-transgenic mice, and then digested with *Pvu*II. One, two, five or 10 copies (15, 30, 75 or 150 pg) of pCXN2 DNA, which contained the human α-galactosidase cDNA, were mixed with non-transgenic mouse genomic DNA before *Pvu*II digestion, and used as positive controls. The digested DNA (10 μg) was separated by electrophoresis on a 1% agarose gel and then transferred to a nitrocellulose membrane (BA85; Schleicher and Schuell, Dassel, Germany). The membrane was hybridized with a ^{32}P-labeled *Pvu*II fragment of human α-galactosidase cDNA (cDNA#7-827) as the probe, as described previously [16].

2.3. Northern blot analysis

Total RNA was extracted from the liver, kidney and heart tissues of transgenic and non-transgenic mice. Poly(A)$^+$RNA was isolated from total RNA using Oligo (dT)-Latex beads (Nippon Roche, Tokyo, Japan) according to the manufacturer's method. Poly(A)$^+$RNA (1 μg) from each tissue was separated by electrophoresis on a 1% agarose gel containing formaldehyde and then blotted onto a nitrocellulose membrane (Schleicher and Schuell). The membrane was hybridized with a ^{32}P-labeled full-length human

α-galactosidase cDNA as a probe using a DNA labeling kit (Nippon Gene, Tokyo, Japan). After the data for α-galactosidase mRNA had been obtained, the membrane was washed and rehybridized with a ^{32}P-labeled human β-actin cDNA (Nippon Gene) to confirm the quality and quantities of samples.

2.4. Enzyme assaying

Homogenates of tissues involving liver, kidney, heart, spleen, small intestine, submaxillary gland, skeletal muscle, cerebrum and cerebellum, and bone marrow cells were centrifuged at $6000 \times g$ for 10 min at 4°C, and the obtained supernatants were used as the samples for enzyme assaying. α-Galactosidase activity was measured with 4-methylumbelliferyl-α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan) as the substrate [17]. The amount of protein was determined with a Bio-Rad dye-binding assay kit (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as the standard.

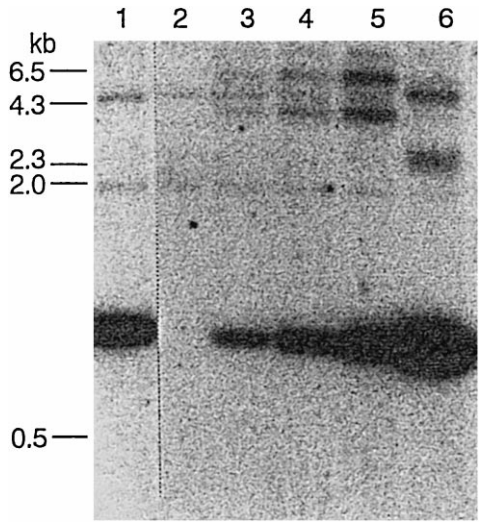

Fig. 1. Southern blot analysis of human α-galactosidase cDNA in the mouse genome. Some 10-μg aliquots of genomic DNAs from the livers of transgenic and non-transgenic mice were digested with *Pvu*II. The digested DNAs were electrophoresed on a 1% agarose gel and then transferred to a nitrocellulose membrane. The membrane was hybridized with a ^{32}P-labeled *Pvu*II fragment of human α-galactosidase cDNA as a probe. The transgene was detected as a 0.8-kb band, and several faint non-specific bands of larger sizes were found. Lane 1, transgenic mouse genomic DNA; lane 2, non-transgenic mouse genomic DNA; lanes 3–6, one, two, five and 10 copies (15, 30, 75, and 150 pg, respectively) of human α-galactosidase cDNA inserted pCXN2 DNAs were mixed with non-transgenic mouse genomic DNA before *Pvu*II digestion, and used as positive controls.

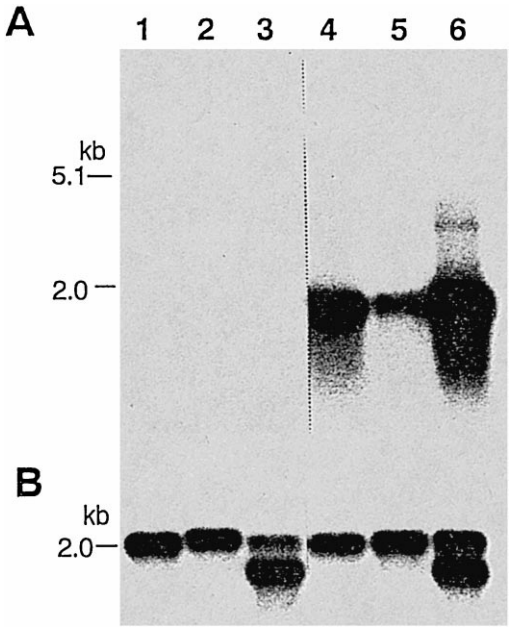

Fig. 2. Northern blot analysis of human α-galactosidase mRNA in mouse tissues. Poly(A)$^+$ RNA (1 μg) was electrophoresed on a 1% agarose gel containing formaldehyde and then transferred to a nitrocellulose membrane. (A) the membrane was hybridized with a ^{32}P-labeled full length human α-galactosidase cDNA; (B) the membrane was rehybridized with a ^{32}P-labeled human β-actin cDNA. Lanes 1–3, poly(A)$^+$ RNAs from a non-transgenic mouse; lanes 4–6, poly (A)$^+$ RNAs from TgN(GLA) 1951. Lanes 1 and 4, liver; lanes 2 and 5, kidney; lanes 3 and 6, heart. The reason why a doublet band reacting with β-actin cDNA was found for the heart tissue is not clear. It might be due to the tissue specificity or the assay conditions with the probe of human origin, but not RNA degradation.

2.5. Western blot analysis

The supernatants of tissue homogenates were subjected to SDS-polyacrylamide gel electrophoresis on a 5–20% gradient gel under reducing conditions according to the method of Laemmli [18]. The samples (liver, 0.5 μg protein; kidney, 3.2 μg protein; and heart, 0.7 μg protein; those of the transgenic mice having α-galactosidase activity of 50 nmol h^{-1}) were transferred from the SDS–polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Bedford, MA, USA). The membrane was incubated for 1 h with rabbit anti-human α-galactosidase antibodies (1:250 dilution) [19], at

Table 1
α-Galactosidase activity in the tissues of transgenic and non-transgenic mice

	α-Galactosidase activity (nmol h^{-1} mg^{-1} protein)	
	TgN(GLA) 1951	Non-transgenic mouse
Liver	53,965	18
Kidney	9177	8
Heart	39,906	4
Spleen	11,056	33
Small intestine	9153	38
Submaxillary gland	30,722	22
Skeletal muscle	33,242	3
Cerebrum	1965	25
Cerebellum	611	27
Bone marrow cells	9354	44
Serum	22,450[a]	14[a]

α-Galactosidase activity: [a]nmol h^{-1} ml^{-1}.

room temperature. Then the membrane was incubated with horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, UK), and the reacted proteins were detected by means of a chemiluminescence detection system (Amersham). The membrane was exposed for 2 min at room temperature to an X-ray film and then developed.

2.6. Immunohistochemistry

The mouse tissues were stored at −80°C until use, and the frozen sections (cut at 10 μm) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 h, and then permeated with 1% Triton X-100 in PBS at 4°C for 18 h. The specimens were incubated with PBS containing 5% (w/v) normal goat serum and 1% (w/v) bovine serum albumin at 4°C for 18 h to block non-specific binding. Subsequently, they were treated with rabbit anti-human α-galactosidase antibodies (1:250 dilution) at 4°C for 72 h, and then finally treated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rabbit IgG (Biosource International, Camarillo, CA, USA) at 4°C for 18 h.

The stained liver and kidney tissues were examined with a confocal laser scanning imaging system (MRC-600; Bio-Rad, Hemel Hempstead, UK) attached to a microscope (Optiphot 2; Nikon, Tokyo, Japan).

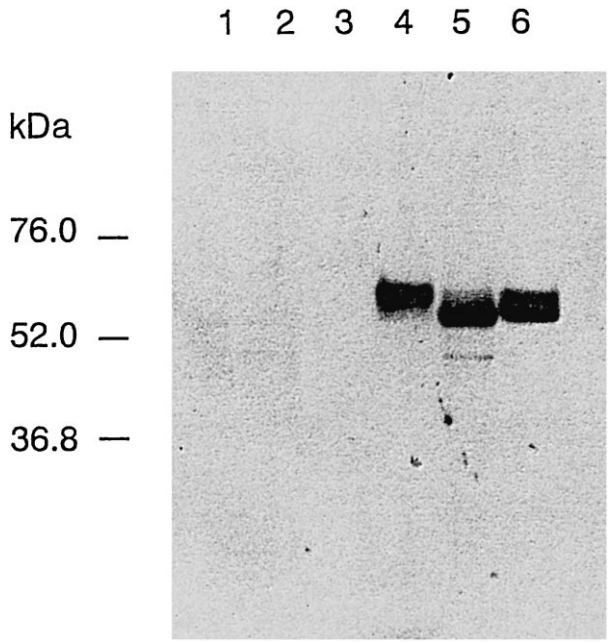

Fig. 3. Western blot analysis of human α-galactosidase in mouse tissues. Extracts of mouse tissues were subjected to SDS-polyacrylamide gel electrophoresis, which was performed on a 5–20% gradient gel under reducing conditions. Then the proteins were electrotransfered to a polyvinylidene difluoride (PVDF) membrane, and the membrane was immunoblotted with rabbit anti-human α-galactosidase antibodies. Lanes 1–3, tissue homogenates from a non-transgenic mouse; lanes 4–6, tissue homogenates from TgN(GLA) 1951. Lanes 1 and 4, liver (0.5 μg protein); lanes 2 and 5, kidney (3.2 μg protein); lanes 3 and 6, heart (0.7 μg protein).

3. Results

3.1. Southern blot analysis

Southern blotting showed the existence of the transgene as a 0.8-kb band in TgN(GLA) 1951 (Fig. 1), and the dose of the transgene integrated was roughly estimated from the density of the corresponding band. The results demonstrated that about five copies of the α-galactosidase cDNA were integrated.

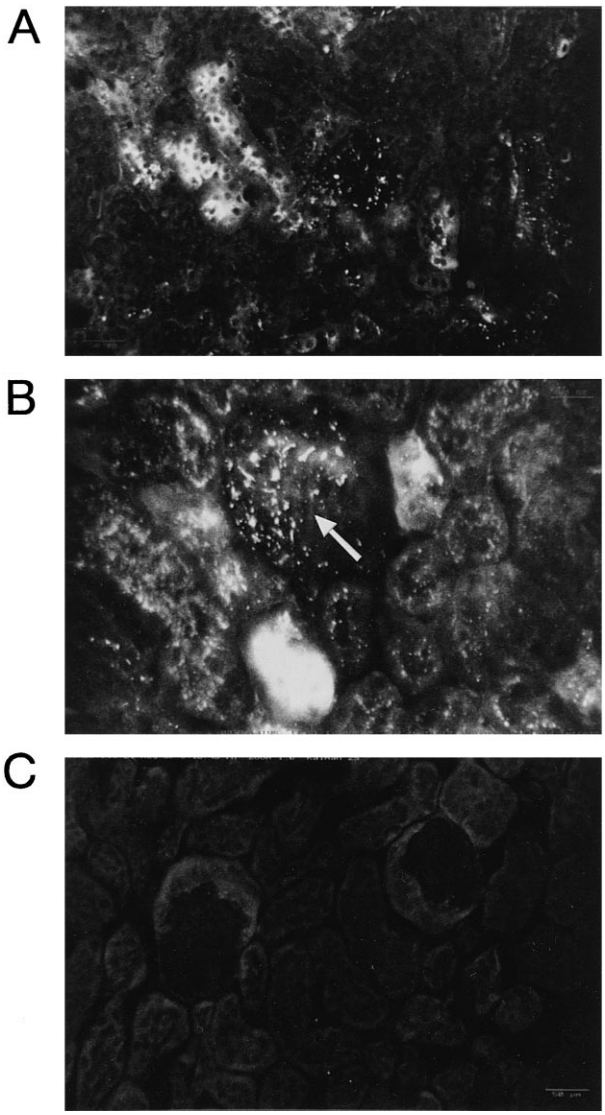

Fig. 4. Indirect immunofluorescence staining of human α-galactosidase in mouse kidney tissues. Tissue slices were fixed with 4% paraformaldehyde in PBS, and then permeated with 1% Triton X-100 in PBS. The specimens were incubated with rabbit anti-human α-galactosidase antibodies, and then treated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rabbit IgG. (A) kidney cortex of TgN(GLA) 1951 ($\times$200); (B) glomerular region (arrow) in kidney cortex of TgN(GLA) 1951 ($\times$400); (C) kidney cortex of a non-transgenic mouse ($\times$200).

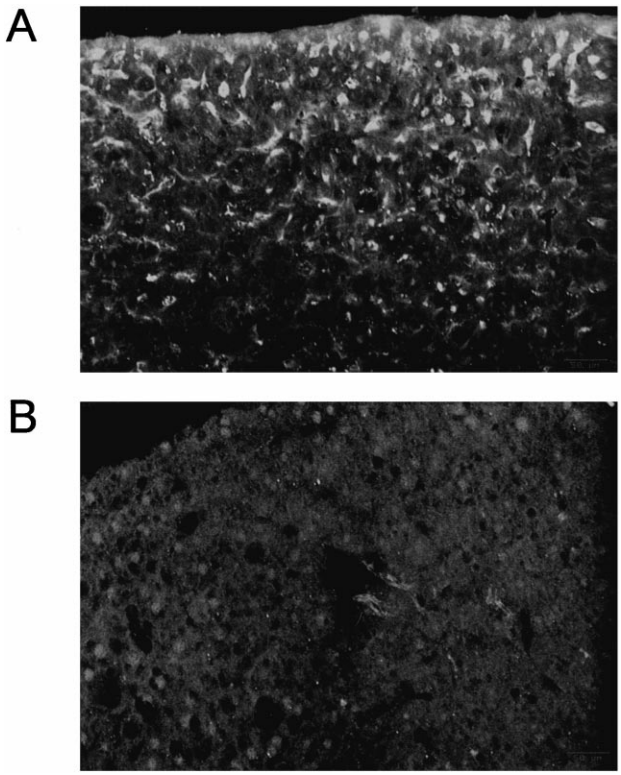

Fig. 5. Indirect immunofluorescence staining of human α-galactosidase in mouse liver tissues. The procedure was described in Fig. 4, with liver tissues as samples. (A) liver tissue of TgN(GLA) 1951 ($\times$200); (B) liver tissue of a non-transgenic mouse ($\times$200).

3.2. Northern blot analysis

The results of Northern blotting showed that the integrated human α-galactosidase cDNA highly transcribed the messages, especially in the heart and liver, followed by in kidney tissue of TgN(GLA) 1951 (Fig. 2).

3.3. Enzyme assay and western blot analysis

The α-galactosidase activity in tissues, bone marrow cells and serum is summarized in Table 1. High enzyme activity was found in these tissues, bone marrow cells and serum of TgN(GLA) 1951, it being about 22 to 11,080-fold higher than in non-transgenic

mice. The high expression of human α-galactosidase in the transgenic mice was also confirmed by Western blotting, in which the expressed enzymes in liver, kidney and heart tissues were detected as 54–60 kDa bands (Fig. 3). The amounts of the expressed products were high in liver and heart tissues, followed by in kidney tissue, which was coincident with the results of enzyme assaying.

3.4. Immunohistochemistry

The distribution of the expressed human α-galactosidase in kidney and liver tissues of TgN(GLA) 1951 was investigated immunohistochemically with a specific antibody by confocal microscopy. Immunofluorescence revealed intense expression of human α-galactosidase in glomerular and tubular cells of kidney tissue (Fig. 4). Strong fluorescence was also found in hepatocytes of the liver, although the immunoreactivity was observed not to be ubiquitous (Fig. 5).

4. Discussion

Transgenic mice expressing human α-galactosidase in many tissues, bone marrow cells and serum were generated, and investigated biochemically and immunohistochemically. Human α-galactosidase was highly expressed in liver and heart, followed by in kidney tissue, which are useful organs for transplantation studies. The molecular sizes of the expressed enzymes in these tissues were estimated to be 54–60 kDa. The differences are predicted to be due to the heterogeneity of sugar chains post-translationally modified in the tissues. In fact, different molecular weights of α-galactosidase in human tissues have been reported (placenta, 57.7 kDa [20]; and spleen, 49.8 kDa [21]).

In this study, we successfully demonstrated the distribution of human α-galactosidase in transgenic mice immunohistochemically. The expressed α-galactosidase protein was clearly identified in glomerular and tubular cells of kidney tissue, and hepatocytes of liver. The transplanted organs could potentially cure Fabry disease by providing the missing enzyme.

Fabry disease represents an irreversible cause of renal failure requiring renal transplantation to correct uremia. Renal [5] and fetal liver [6] transplantation has been performed, expecting also to provide of the missing enzyme, α-galactosidase. Recently improved techniques have facilitated successful renal transplantation with a high graft survival rate [9–11]. But there have been few reports of late graft histology [22], because it is difficult to obtain recurrent biopsied tissues in human subjects. These transgenic mice overexpressing human α-galactosidase will be useful as a donor for transplantation to non-transgenic mice. It enables recurrent biopsies, long-term observation, and chase study of α-galactosidase originated from a transplanted organ in a recipient. Recently, Ohshima et al. [23] established α-galactosidase gene knock-out mice, and showed that they are useful as a Fabry disease model. Organ transplantation from the transgenic mice to such knock-out mice will especially provide important information for those in human subjects.

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